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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classificati n 6 :
C12N 9/00, 9/10, 15/00, 15/09, 15/29, 15/64, 15/82, A01H 1/00, 4/00, 5/00

A1

(11) International Publication Number: WO 97/35960

(43) International Publication Date: 2 October 1997 (02.10.97)

(21) International Application Number: PCT/US97/04982

(22) International Filing Date: 24 March 1997 (24.03.97)

(30) Priority Data:
08/622,679 26 March 1996 (26.03.96) US

(71) Applicant: UNIVERSITY OF HAWAII [US/US]; 2800 Woodlawn Drive, Honolulu, HI 96822 (US).

(72) Inventors: STILES, John, I.; 46-372 Kumoo Loop, Kaneohe, HI 96744 (US). MOISYADI, Istefo; Apartment 3213, 1717 Mott-Smith Drive, Honolulu, HI 96822 (US). NEUPANE, Kabi, Raj; 2724 Kolo Place, Honolulu, HI 96826 (US).

(74) Agent: MEYER, David, J.; Graham & James L.L.P., 14th floor, 801 South Figueroa Street, Los Angeles, CA 90017-5554 (US).

(81) Designated States: JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: PURIFIED PROTEINS, RECOMBINANT DNA SEQUENCES AND PROCESSES FOR PRODUCING CAFFEINE FREE BEVERAGES

(57) Abstract

Purified proteins, DNA sequences that code on expression therefore and recombinant DNA molecules, including hosts transformed therewith, for transforming coffee plants to suppress the expression of caffeine. The DNA sequences and recombinant DNA molecules are characterized in that they code on expression for an enzyme in the pathway for caffeine synthesis in coffee. Coffee plants transformed with DNA molecules that code on transcription for mRNA that is antisens to mRNA that codes on expression for at least one enzyme in the pathway for caffeine biosynthesis.

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PURIFIED PROTEINS, RECOMBINANT DNA SEQUENCES AND PROCESSES FOR PRODUCING CAFFEINE FREE BEVERAGES

This application relates to purified proteins, recombinant DNA sequences, hosts transformed therewith and processes for producing caffeine-free beverages and food products. More particularly, this application relates to purified proteins, and recombinant DNA sequences that suppress the expression of caffeine in coffee plants, and in fruit harvested therefrom. The invention produces stable lines of caffeine free coffee plants whose fruit, after roasting and grinding, can be used to prepare caffeine free coffee. It is expected that the invention can be used to suppress caffeine synthesis in tea (Camellia sinensis) and cola (Cola acuminata), as well as related alkaloids in chocolate (Theobroma cacao).

BACKGROUND OF THE INVENTION

Coffee is prepared from the roasted ground beans of the plants of the genus Coffea, generally from the species C. arabica. Coffee plants produce the alkaloid caffeine, which is present in their dried fruit, coffee beans. Because many coffee drinkers prefer coffee without caffeine, a number of processes have been developed to remove caffeine from coffee beans. All of these processes result in the removal of substances other than caffeine from the beans, thereby adversely affecting the taste of coffee brewed from the treated beans. Although a few naturally occurring caffeine free coffees and related genera are known (Mascarocoffea spp. and Coffea bengalensis), they have no commercial value. (Charrier and Berthaud, "Variation Of Caffeine Content In The Coffea Genus", Cafe' Cacao The', 14:251-264 (1975)). Accordingly, there is a need for a method for producing decaffeinated coffee beans that does not result in the removal of substances from the beans other than caffeine.

Caffeine is a naturally occurring purine alkaloid produced by coffee and tea plants, among others. It is believed that caffeine synthesis protects the plants from insects. Coffee plants synthesize caffeine from the nucleoside xanthosine in four sequential reactions as

shown in Figure 1. For review see Suzuki, T., Ashihara, H. and Waller, G.R., Phytochemistry 31:2575 (1992). The first step in the pathway is the methylation of the nucleoside xanthosine by S-adenosylmethionine, which is catalyzed by the enzyme xanthosine N^7 methyl transferase (XMT). The product, 7-methylxanthosine is hydrolyzed (a ribose is removed) to 7-methylxanthine, and undergoes further methylations to theobromine and caffeine. It is to be expected that interruption of this sequence of synthetic reactions would block caffeine synthesis.

Accordingly, a strategy for selectively eliminating caffeine from coffee plants is to prevent synthesis of specific enzymes in the pathway for caffeine biosynthesis. In one embodiment this invention relates to genetic alteration of coffee plants to eliminate synthesis of XMT. In the presently preferred embodiment, synthesis of XMT is suppressed by transforming coffee plants with a DNA sequence that codes on transcription for a messenger RNA (mRNA) that is antisense to the mRNA that codes on expression for XMT. The invention may be generalized to produce other caffeine free beverages and food products, including tea, cocoa, and other chocolate-based beverages or foods.

SUMMARY OF INVENTION

Purified proteins, DNA sequences that code on expression therefore and recombinant DNA molecules, including hosts transformed therewith, for transforming coffee plants to suppress the expression of caffeine. The DNA sequences and recombinant DNA molecules are characterized in that they code on expression for an enzyme, xanthosine N^7 methyl transferase (XMT), that is the first step in the pathway for caffeine synthesis in coffee. The base sequence of that DNA and the predicted amino acid sequence of XMT is provided.

Coffee plants are transformed with DNA molecules that code on transcription for mRNA that is antisense to mRNA that codes on expression for at least one enzyme in the pathway for caffeine biosynthesis. The antisense RNA binds to XMT mRNA, thereby inactivating the mRNA encoding the first step in the pathway for caffeine synthesis. The

result is that the transformed plants are incapable of synthesizing caffeine, though other aspects of their metabolism is not affected.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic drawing of the pathway for caffeine synthesis in Coffea arabica.

Figure 2 is a photograph of a silver stained SDS PAGE gel of purified xanthosine N^7 methyl transferase.

Figure 3 is a densitometric plot showing elution of tryptic fragments of purified xanthosine N^7 methyl transferase following HPLC separation.

Figure 4 is a description of the oligonucleotide primers used to screen the cDNA library cDNA encoding xanthosine N^7 methyl transferase.

Figure 5 is the base sequence of the cDNA that encodes xanthosine N^7 methyl transferase.

Figure 6 with the predicted amino acid sequence of xanthosine N^7 methyl transferase.

DETAILED DESCRIPTION OF THE INVENTION

In order that the invention herein described may be more fully understood, the following detailed description is set forth. In the description the following terms are employed:

Nucleotide -- A monomeric unit of DNA or RNA consisting of a sugar moiety (pentose), a phosphate, and a nitrogenous heterocyclic base. The base is linked to the sugar moiety via the glycosidic carbon (1' carbon of the pentose) and that combination of base and

sugar is called a nucleoside. The base characterizes the nucleotide. The four DNA bases are adenine ("A"), guanine ("G"), cytosine ("C"), and thymine ("T"). The four RNA bases are A, G, C, and uracil ("U").

DNA Sequence -- A linear array of nucleotides connected one to the other by phosphodiester bonds between the 3' and 5' carbons of adjacent pentoses.

Codon -- A DNA sequence of three nucleotides (a triplet) which encodes through mRNA an amino acid, a translation start signal or a translation termination signal. For example, the nucleotide triplets TTA, TTG, CTT, CTC, CTA and CTG encode for the amino acid leucine ("Leu"), TAG, TAA and TGA are translation stop signals and ATG is a translation start signal, which also encodes the amino acid methionine ("MET").

Polypeptide -- A linear array of amino acids connected one to the other by peptide bonds between the amino and carboxy groups of adjacent amino acids.

Genome -- The entire DNA of a cell or a virus. It includes inter alia the structural gene coding for the polypeptides of the substance, as well as promoter, transcription and translation initiation and termination sites.

Gene -- A DNA sequence which encodes through its template or messenger RNA ("mRNA") a sequence of amino acids characteristic of a specific polypeptide.

Transcription -- The process of producing mRNA from a gene or DNA sequence.

Translation -- The process of producing a polypeptide from mRNA.

Expression -- The process undergone by a gene or DNA sequence to produce a polypeptide. It is a combination of transcription and translation.

Plasmid -- A nonchromosomal double-stranded DNA sequence comprising an intact "replicon" such that the plasmid is replicated in a host cell. When the plasmid is placed

within a unicellular organism, the characteristics of that organism may be changed or transformed as a result of the DNA of the plasmid. For example, a plasmid carrying the gene for tetracycline resistance (TETR) transforms a cell previously sensitive to tetracycline into one which is resistant to it. A cell transformed by a plasmid is called a "transformant."

<u>Phage or Bacteriophage</u> -- Bacterial virus many of which consist of DNA sequences encapsidated in a protein envelope or coat ("capsid").

<u>Cloning Vehicle</u> -- A plasmid, phage DNA, cosmid or other DNA sequence which is able to replicate in a host cell, characterized by one or a small number of endonuclease recognition sites at which such DNA sequences may be cut in a determinable fashion without attendant loss of an essential biological function of the DNA, e.g., replication, production of coat proteins or loss of promoter or binding sites and which contain a marker suitable for use in the identification of transformed cells, e.g., tetracycline resistance or ampicillin resistance. A cloning vehicle is often called a vector.

<u>Cloning</u> -- The process of obtaining a population of organisms or DNA sequences derived from one such organism or sequence by asexual reproduction.

<u>Recombinant DNA Molecule or Hybrid DNA</u> - A molecule consisting of segments of DNA from different genomes which have been joined end-to-end outside of living cells and able to be maintained in living cells.

<u>cDNA</u> - A DNA strand complementary to an mRNA that codes for a particular polypeptide.

Although the strategy for producing caffeine free coffee may be generalized to other enzymes in the pathway for caffeine synthesis in coffee and other caffeine producing plants, in the presently preferred embodiment of this invention, the expression of the first unique enzyme in the pathway, xanthosine N^7 methyl transfersase (XMT) is suppressed. While the role of XMT in caffeine synthesis has been elucidated by radiolabeling of precursors, to date

the enzyme has not been purified nor has its amino acid sequence been determined. This invention therefore includes substantially purified XMT. The invention further includes the amino acid sequence of tryptic fragments isolated from the purified XMT.

cDNA probes based on portions the amino acid sequence obtained from samples of the purified enzyme were synthesized and a portion of the gene was amplified using PCR. The PCR products were used to screen a cDNA library synthesized from young leaf mRNA to identify transcripts encoding XMT. The positive transcripts were sequenced and approximately 90% of the gene encoding XMT was obtained.

DNA that codes on expression for XMT are incorporated into a pBI-121 transformation vector which includes a kanamycin resistance gene. Successful incorporation of the vectar into plant cells will be monitored by acquisition of antibiotic resistance. The constructs are used to transform coffee somatic embryos in tissue culture. The transformed embryos are thereafter grown into novel coffee plants that do not produce caffeine. Naturally decaffeinated coffee is prepared from roasted ground fruit from these novel plants.

More specifically, fresh leaf tissue from young leaves of C. arabica was macerated and protein extracted therefrom. Column purified extracts were assayed for enzymatic activity, by monitoring the methylation of xanthosine using C^{14} labeled S-adenosylmethionine as substrate. The reaction product was confirmed as 7-methylxanthosine by comparing the migration of the labeled reaction product with migration of 3-methylxanthine, 7-methylxanthine, 8-methylxanthine, 7-methylxanthosine, xanthine and xanthosine in each of four different chromatography systems.

The purity of the protein isolates was determined using SDS PAGE electrophoresis and two dimensional gel electrophoresis. Silver staining of one dimensional SDS PAGE gels indicated the presence of a doublet with the enzymatic activity of XMT, with a molecular weight of 36-37 kiloDaltons (kD) as shown in Figure 2. Each protein was further resolved

with isoelectric focusing. The data indicates the presence of isozymes of XMT that may result from post translational modification of the protein; alternatively, there may be a gene family encoding XMT enzymes.

The doublet visualized on SDS PAGE gels was used for protein sequencing. Purified XMT was subjected to partial tryptic digestion to create fragments for further analysis; three peaks were resolved using HPLC. Sequencing was performed by the Protein Structure Laboratory of the University of California, Davis using automated Edman degradation. (Edman, P. and Begg, G., Eur. J. Biochem. 1:80). Two unique sequences were resolved, and used to construct primers for probe synthesis. RNA was extracted from coffee leaves. mRNA containing poly (A^+) sequences was purified therefrom. A cDNA library was prepared from the poly (A^+) mRNA using reverse transcriptase. Double stranded DNA was prepared using DNA polymerase I, and recovered by precipitation. The cDNA was fractionated and inserted into phage for amplification. The cDNA library was screened with a PCR synthesized probe produced using primers based on the DNA sequence expected from the amino acid sequence of the purified XMT. A clone producing a cDNA containing all of the sequences encoding XMT has been identified.

The cDNA corresponding to the gene encoding XMT is used to transform embryonic coffee plants. The plasmid pBI-121 is used as a transforming vector. The sequences corresponding to DNA that codes on expression for XMT is inserted into the plasmid in an inverted orientation adjacent to a cauliflower mosaic virus 35S promoter. RNA transcribed therefrom will be complementary to mRNA that encodes the amino acid sequence of XMT. Complete constructs are amplified in bacterial hosts. The hosts are disrupted and the amplified vector is attached to colloidal gold particles. The gold particles with adherent vectors are inserted into coffee plant protoplasts by propelling the particles at high speed at the cells as described in U.S. patent 5,107,065. Young plants successfully transformed are identified by antibiotic resistance. The transformed plants do not produce caffeine.

EXAMPLES

A. Purification of xanthosine-N^7-methyltransferase from *C. arabica* L. cv Guatemalan coffee leaves.

Young leaf tissue, less than 5 mm in length (equivalent to the B3 stage (Frischknecht, P.M., Ulmer-Dufek, J. and Baumann, T.W. (1986) Phytochemistry 25:613) were collected from trees grown at the University of Hawaii Waimanalo Research Station, Oahu, Hawaii. Leaves were immediately immersed in liquid nitrogen (liquid N_2) and stored at -70°C until used. All subsequent procedures were carried out at 4°C unless otherwise stated. Leaf tissue (150 g) was macerated in a mortar and pestle under liquid N_2 and, while still frozen, transferred to a pre-chilled domestic coffee grinder and ground with a small piece of dry ice for about 30 sec. The powdered tissue was added to a beaker containing 1.5 L of ice cold 80% acetone, 5 mM thiourea, and 12.5 mM β-mercaptoethanol. After mixing on a magnetic stirrer for 45 min, the tissue was recovered by filtration under vacuum in a Buchner funnel containing Whatman No. 1 filter paper. The tissue was washed with 2.5 L of 80% ice cold acetone containing thiourea and β-mercaptoethanol as above, air dried for 20 min and then lyophilized for 48 hours.

The resulting acetone powder was homogenized in a blender with 400 mL of extraction buffer (EB) (0.1 M PIPES [pH 7.0], 0.5 mM Na_2EDTA, 0.5 mM Na_2EGTA, 5% ascorbic acid, 5 mM dithiothreitol [DTT], 5 mM thiourea, 12 mM L-cysteine HCl, 1% polyethylene glycol (PEG) 20,000, 0.1 mM phenylmethylsulfonyl fluoride [PMSF], and 20 g polyvinyl-polypyrrolidone [PVPP]). The slurry was homogenized for 10 min at medium speed, and then transferred into 250 mL centrifuge bottles and centrifuged at 23,000x*g* for 30 min in a GSA (Dupont-Sorvall) rotor.

The 350 mL crude supernatant obtained was brought to 40% ammonium sulfate (AS) saturation over 30 min by the slow addition of 79.86 g AS powder while being stirred in a beaker surrounded by an ice bath. The mixture was once again transferred to 250 mL centrifuge bottles and centrifuged at 23,000x*g* for 30 min as above. The 350 mL supernatant

obtained was loaded into a 40 mL Macro-Prep (Bio-Rad) methyl hydrophobic interaction chromatography (HIC) column at the flow rate of 2.5 mL/min. All column fractions were monitored for protein using absorbance at 280 nm. The HIC column was washed with pre-equilibration buffer containing 1.7 M AS, 20 mM bis-tris-propane (pH 6.8), and 5 mM DTT until a baseline near zero was established. The column was then stripped with a buffer containing 10 mM tris (pH 7.0), 5 mM DTT, 1 mM $MgCl_2$. The first 15 mL out of the column was discarded and the remaining eluate (200 mL) was loaded under gravity into a 100 mL Affi-Gel blue affinity gel (100-200 mesh, Bio-Rad) column that had the dye Cibacron blue F3GA covalently attached to the matrix. The gel was pre-equilibrated with 10 mM tris (pH 7.0), 5 mM DTT, 1 mM $MgCl_2$ loading buffer. The column was washed extensively with this loading buffer until the baseline stabilized near zero, and the bound proteins were eluted with a buffer containing 10 mM tris (pH 7.0), 5mM DTT, and 1.5 M sodium chloride (NaCl).

The 142 mL Affi-Gel Blue Gel column eluate was made 1.7 M AS by the slow addition of 31.8 g AS powder while being stirred for 30 min in a beaker surrounded by an ice bath. The slurry was centrifuged in 250 mL centrifuge bottles at 23,000xg for 30 min as above, and the supernatant loaded into an FPLC Phenyl-Sepharose column XK 26/20 (Pharmacia) at 23°C. The column was pre-equilibrated with a buffer containing 20 mM bis-Tris-Propane (pH 6.8), 5 mM DTT, and 1.7 M AS. When a baseline was established near zero the proteins were eluted out of the column in a 40 min reverse gradient of 1.7 M AS to 0 M AS at a flow rate of 5 mL/min, collecting 1 min fractions. The 0 M AS elution buffer contained 10 mM tris (pH 7.0), 5 mM DTT, and 1 mM $MgCl_2$.

Activity assays on the fractions collected indicated that the majority of enzymic activity for xanthosine-N^7-methyltransferase was concentrated in fractions 49 to 54. These fractions were pooled into 30 mL final volume, and then loaded into a 6 mL ATP-agarose column (Sigma Chemicals, A2767) by gravity at 4°C. The column was pre-equilibrated with 10 mM tris (pH 7.0), 5 mM DTT, and 1 mM $MgCl_2$. After stabilization of the baseline, the

column was stripped with 20 mL of pre-equilibration buffer containing 100 μM xanthosine, and washed with an additional 40 mL pre-equilibration buffer. Both column eluates were pooled and loaded into a Mono-P HR 5/20 FPLC (Pharmacia) column pre-equilibrated with 25 mM bis-tris (pH 6.0) and 9% betaine at 23°C. After the baseline stabilized the column was eluted with 100 mL Polybuffer 74 (10 mL:90 mL H_2O, v:v) (pH 4.0) (Pharmacia), and 9% betaine at a flow rate of 1 mL/min. The collection tubes contained 100 μL 0.5 M tricine buffer (pH 7.0), and 50 mM DTT to give a final concentration in 1 mL of 50 mM tricine (pH 7.0), and 5 mM DTT in 1 min fractions. This in effect stabilized the final pH conditions for the proteins eluted under slightly acidic pH from the Mono-P column. The major activity for xanthosine-N^7-methyltransferase in collection tubes without tricine was found in fractions 15 and 16 of the gradient eluting from the column with a pH of 5.42 and 5.35 respectively. It was important not to freeze the protein samples at any stage of the purification, as this had a substantial negative effect on the activity state of xanthosine-N^7-methyltransferase.

B. Assay of enzyme activity.

The 100 μL standard assay mixture contained 50 mM tricine (pH 7.0), 1200 μM xanthosine, 5 mM DTT, 7.5 μM *S*-adenosyl-L-[methyl-^{14}C]-methionine (SAM) (60mCi/mmol; DuPont NEN), and 1 mM Na_2EDTA. The reaction mixture (50 μL without enzyme) was preincubated for 10 min at 25°C and the reaction was initiated by the addition of 50 μL enzyme solution and allowed to proceed at 25°C for 1 hour. At the end of the incubation period three 30 μL aliquots of the reaction were removed and terminated by adding to 8 μL of 0.6 M perchloric acid ($HClO_4$). The same was done for zero time controls in order to detect true enzymic activity. This mixture was centrifuged in a microcentrifuge for 5 min and 19 μL of the supernatant was mixed with 1.0 μL of 33 mM 7-methyl-xanthosine. These mixtures were spotted on Whatman No.1 chromatography paper and developed with n-butanol-acetic acid-$H_2$0 (*n*-BuOH-HOAc-H_2O) (4:1:1). The position of 7-methylxanthosine was determined by its blue fluorescence when exposed to short

wavelength UV light. This region was cut out of the chromatograms and the radioactivity was determined by scintillation counting using 3 mL Scinti-verse scintillation fluid (Fisher Scientific). Counting efficiency was 74.7%. Background and non-specific radiation detected in the 7-methylxanthosine region of the zero time samples were subtracted.

C. Identification of the reaction product.

The site of methylation on the xanthine ring was identified by hydrolysis of the sugar from the methylated xanthosine reaction product and separation in 4 different chromatography systems. The product from two 100 μL reactions done as described above and containing 6 μL of 33 mM 7-methylxanthosine as carrier, was applied as a band at the origin of a Whatman No.1 paper chromatogram. The chromatogram was developed in *n*-BuOH-HOAc-H_2O (4:1:1). The region of the chromatogram corresponding to methylated xanthosine was detected as above, cut into small pieces, placed in a sterile tube, and incubated with 35 mL of deionized water at 37°C with shaking overnight. The extract was filtered through 2 layers of miracloth followed by a 0.22 μm filter and then lyophilized. The dried extract was resuspended in 1.0 mL of deionized water, placed in a glass digestion vial and lyophilized. The sample was resuspended in 400 μL of 1.0 M HCl and incubated for 1 hour at 100°C. The digest was lyophilized, resuspended in 400 μL of 3 mM 7-methyl-xanthine and again lyophilized. The digest was resuspended in 40 μL of deionized water, and 10 μL was chromatographed in each of four different systems. 1-Methylxanthine, 3-methylxanthine, 7-methylxanthine, 8-methylxanthine, 7-methylxanthosine, xanthine and xanthosine were included on each chromatogram for comparison. The following chromatography systems were used; Whatman No.1 paper developed in *n*-BuOH-HOAc-H_2O (4:1:1) and C8 thin layer plates (Whatman KC18F) developed in either isoamyl alcohol-H_2O-acetonitrile (41:4:5), ethanol-H_2O (4:1) or *tert*-BuOH-HOAc-H_2O (4:1:1). After drying, the chromatograms were sprayed with En^3Hance (Dupont NEN), redried and exposed for 30 days to pre-flashed Fuji RX_{GCU} X-ray film at -70°C.

D. Identification of proteins by gel electrophoresis.

Extracts obtained as above were used in single dimension (1D) SDS-PAGE minigels (main gel:12.5% acrylamide, 0.8% methylene bisacrylamide; stacking gel:7.5% acrylamide, 0.21% methylene bisacrylamide) by mixing with Laemmli sample buffer (Laemmli, U.K., Nature 227:680 (1970)), and in two-dimensional (2D) mini IEF/SDS-PAGE by the modified method of O'Farrell et.al. (O'Farrell, P.Z., Goodman, H.M., O'Farrell P.H., Cell 12:1133 (1977)). Two-dimensional electrophoresis was made possible by precipitating proteins with 50 volumes of 100% ethanol for 1 hour and redissolving the proteins in isoelectric focusing (IEF) sample buffer containing 5% ampholines (1:1, v:v, pH 3-10:pH 5-7, LKB-Pharmacia). The ratio of the original protein extract to the IEF sample buffer was maintained at least 1:2 to ensure that any remaining buffer constituents from the chromatography steps did not interfere with IEF. Equal total protein samples (<20 μg) were applied to the basic end of prefocused tube gels (8.8% acrylamide, 1.6% methylene bisacrylamide) containing 5% ampholines as above. The gels were focused for 10,000V-hours plus an additional 2 hours at 1,000 V. Blank focused gels were cut into 5 mm sections and incubated in 0.5 mL of 100 mM $CaCl_2$ for 24 hours, and the pH of the segments was determined. From this analysis, the pH gradient of the IEF gel was estimated to range from 4.4 to 6.0.

The tube gels were prepared for SDS-PAGE by a brief H_2O wash followed by three washes (10 min each) in hot Laemmli sample buffer. The tube gels were placed on the top of SDS-PAGE gels (main gel:12.5% acrylamide, 0.8% methylene bisacrylamide; stacking gel:7.5% acrylamide, 0.21% methylene bisacrylamide) and held in place with 3% agarose in Laemmli sample buffer. Proteins were visualized by silver-staining. In 1D gels the Mono-P fraction 16 which had the highest enzymic activity indicated only the presence of a doublet under silver staining (Figure 2). The molecular weights of these proteins (kD) were approximately 37.6 and 36.1 kD. In 2D gels each protein separated into two spots. The isoelectric point (IP) of the more acidic one had an average value over several gels of 5.2,

and the more basic one of 5.3. Their molecular weight's however now averaged 43.5 kD, with the upper and lower peptides fusing into each other. Therefore, there is a distinct difference in kD between 1D and 2D gels. The similar migration of all these four peptides in Mono-P columns, 1D and 2D gels indicates that they are isozymes which may be post-translationally modified. Alternatively they may be products of a gene family which have slight differences in their structure from each other, resulting in the differing isozymes observed.

E. Protein sequencing.

Total protein estimation by the procedure of Lowry (Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., J. Biol. Chem. 193:265 (1951)) for fraction 16 of Mono-P indicated there was a total of 100 μg of protein in the 1 mL fraction. It is our experience that at these low concentrations of protein, Lowry values tend to be an over-estimation of the actual amount present. We decided to overcompensate for this by using a substantial part of this fraction for protein sequencing. A 900 μL portion of Mono-P fraction 16 representing 90 μg was placed in a sterile 1.5 mL microcentrifuge tube and 216 μL of 100% trichloro-acetic acid (TCA) was added to it. After mixing, the tube was allowed to incubate on ice overnight, and was then centrifuged at 14,000 rpm in a microcentrifuge for 30 min at 4°C. The supernatant was removed by aspiration, and the pellet washed twice with 1 mL of 75% ethanol, each washing being followed by a centrifugation step. The pellet was dried by placing the tube in a speedvac and spinning for 1 min under vacuum. The precipitate had 20 μL of 2 x Laemmli sample buffer added to it. It was then boiled in a water bath for 5 min, and then microfuged for 1 min. When the tube temperature had cooled down to 23°C the whole amount was loaded into a single lane of a 12.5% 1D gel. At the termination of electrophoresis proteins were visualized by staining with 0.1% Coomassie R-250 in aqueous 50% methanol and 10% acetic acid, (w:v:v), and then destained. The same doublet of 37.6 and 36.1 kD observed in silver stained gels was also visible in the Coomassie stained gels.

The region of the gel comprising this doublet was cut out and used for protein sequencing by automated Edman degradation.

Protein sequencing was performed by the University of California, Davis, Protein Structure Laboratory standard protocol. The gel piece containing the doublet was washed 4 times with 15 mL of H_2O by shaking gently for 15 min to remove the acetic acid and SDS remaining from the previous steps. The gel piece was diced with a razor blade to 2 mm squares, and transferred to a 1.5 mL microcentrifuge tube. The gel pieces were dehydrated in a Speed-Vac for 2 hours until they did not adhere to the tube. Next 30 µL of gel rehydration buffer (0.1 M Tris-HCl, pH 9.0, 0.05% SDS) was added, and the pH verified at 8.0 by spotting 0.5 µL on pH paper. The digestion enzyme Lys-C (0.2 ug) from *Achromobacter lyticus* (Wako) was added, along with additional rehydration buffer to completely hydrate the gel pieces and leave a little extra buffer. The mixture was allowed to incubate overnight at 30°C. After the incubation period, the supernatant was removed to a fresh, sterile microcentrifuge tube and stored. Enough water was added to cover the gel pieces, and they were incubated for a further 2 hours at 30°C. The supernatant was removed and stored in the same microcentrifuge as before. This wash step was repeated once more, with the supernatants being combined with the previous two washes. The gel pieces were then covered with a solution comprising of 0.1% trifluoroacetic acid (TFA) in 80% acetonitrile, and incubated for 1 hour at 30°C. The supernatant was collected and added to the tube containing all the previous supernatants. The last wash was repeated once more, and the pooled supernatants were dried in a speed-vac.

The dried tryptic digestion products were dissolved in 25 µL of 6 M guanidine-HCl, 0.4 M tris (pH 8.2), and the pH verified by spotting 0.5 µL on pH paper. One µL of 450 mM DTT was added and the digest was incubated for 45 min at 50°C. After cooling to room temperature 2 µL of 500 mM iodoacetamide was added, and incubated for a further 15 min at 23°C. At the end of this incubation 72 µL of water was added to give a final concentration of 1.5 M guanidine, and 0.1 M tris. The sample was then centrifuged for 5 min at 14,000 rpm in

a microcentrifuge and the supernatant was carefully removed to a new microcentrifuge tube. To the precipitated pellet 25 μL of 0.1% TFA vas added and vortexed. The tube was then re-centrifuged as b fore, and the supernatant added to that from the previous step.

The cleavage fragments from the tryptic digestion were resolved from each other by capillary high pressure liquid chromatography (HPLC) in a C18 1 mm x 10 cm column, utilizing a linear gradient over 90 min of 5% solvent A (0.1% TFA) to 70% solvent B (0.075% acetonitrile) at a flow rate 100 μL per min. The UV detection was set at 210 nm with the scale ranging from 0 to 0.1 A. The recovery of individual peaks indicated the presence of several distinct peptides as shown in Figure 3. As a control a portion of the original BDS-PAGE gel that did not contain protein was carried through the digestion process. The filled peaks shown in Figure 3 were common between this control and the sample. The 3 peaks labelled A, B, and C were subjected to automated Edman degradation. Two of the peaks (A and B) yielded overlapping unique sequences representing the same protein fragment (Figure 2, Fragments A and B). The third peak (C) yielded a different unique sequence (Figure 2, Fragment C).

F. Synthesis of oligonucleotide DNA primers for xanthosine-N^7-methyltransferase.

Chemical synthesis of 20 mer primers for the two amino acid sequences obtained by the digestion fragments of xanthosine-N^7-methyltransferase was done by The Midland Certified Reagent Company. Regions of the fragments selected had minimal nucleic acid degeneracy, and where possible amino acids that have extensive genetic code redundancy were avoided. Where this was not possible more than one primer was synthesized for the same fragment to include all of the possible alternative codon combinations. Furthermore, we also synthesized primers such that they were complementary to the coding strand of the DNA sequences which code for the amino acid sequence. Third position nucleotide degeneracies of three or more were overcome by using inosine at thse positions. Where the

degeneracy of a nucleotide was two-fold, both nucleotides were included in primer synthesis (Figure 3).

G. Extraction of RNA from B3 stage young coffee leaves.

All items used during the extraction were sterile, RNase-free, and prepared by treating with 0.1% DEPC water. All centrifugation steps were carried out at 4°C unless otherwise stated.

Young coffee leaves of the B3 stage were collected and stored as previously described. Total RNA was isolated from 100 g of this young leaf tissue by grinding under liquid nitrogen and immediately transferring into a prechilled domestic coffee grinder. The tissue was ground to powder together with a small piece of dry-ice. The tissue was then added to 200 mL of homogenization buffer made up of 100 mM tris-HCl (pH 9.0), 200 mM NaCl, 15 mM Na_2EDTA, 0.5% sarcosyl, and freshly added 100 mM β-mercaptoethanol. To this was added 200 mL buffer-equilibrated phenol, and 40 mL of a mixture of chloroform:isoamyl alcohol (24:1, v:v). The tissue was then homogenized in a glass beaker in an ice bath for 2 min at high speed in a Polytron homogenizer. Immediately after homogenization 14 mL 3 M sodium acetate (pH 4.0) was added and mixed by operating the homogenizer for an additional 1 min. The homogenate was then stored on ice for 15 min., and subsequently transferred into two 250 mL polypropylene centrifuge tubes. Centrifugation was performed in a GSA (DuPont Sorvall) rotor at 16,000x*g* for 10 min. The aqueous phase (top layer) was transferred to a new 250 mL polypropylene centrifuge tube and an equal volume of isopropanol was added to it.

This mixture was incubated overnight at -20°C and then centrifuged at 10,000x*g* for 10 min to collect the precipitated RNA.

The RNA pellet was washed with 70% ethanol and re-centrifuged at 10,000x*g* for 5 min. The ethanol was decanted and the pellet dried under vacuum for 5 min. The pellet was then resuspended in 15 mL of DEPC-treated water. The RNA suspension was transferred into a sterile 40 mL screw-cap centrifuge tube and the insoluble material removed by centrifugation at 10,000x*g* for 5 min. The supernatant was transferred to a new 40 mL screw-cap centrifuge tube and 5 mL of 8 M LiCl was added to it to give a final concentration of 2 M LiCl. The tube was incubated overnight at 4°C and the RNA was recovered by centrifugation at 14,000x*g* for 10 min. The RNA pellet was then washed with 70% ethanol, centrifuged at 10,000x*g* for 5 min, and briefly dried under vacuum. The pellet was resuspended in 5 mL DEPC-treated water and centrifuged at 10,000x*g* for 5 min to remove insoluble material. The supernatant was transferred into four sterile 1.5 mL microcentrifuge tubes and stored on ice. The quantitation of 10 µL of the total RNA solution in a Shimadzu UV 160U spectrophotometer in a 230 to 330 nm spectrum indicated that there was 42.8 mg of RNA. The tubes containing the RNA were stored at -70°C.

H. Purification of poly (A^+) mRNA from total RNA.

The total RNA preparation was enriched for poly (A^+) RNA (mRNA) using the PolyATtract II mRNA isolation system kit (Promega Corporation). A 600 µL aliquot of the total RNA equalling 5.1 mg was added into a tube of the above mentioned kit and made to 2.43 mL final volume with RNase-free water. After heating at 65°C for 10 min, 10 µL of 50 pmole/ml biotinylated oligo(dT) and 60 µL of 20x SSC (175.3 g/L NaCl, 88.2 g/L sodium citrate, pH 7.0) were added and the mixture was allowed to slowly cool to room temperature over a period of approximately 30 min. An aliquot of the streptavidin paramagnetic particles were washed 3 times in 0.5x SSC (1.5 µL per wash) and resuspended in 0.5 mL of 0.5 x SSC. The RNA solution containing the biotinylated oligo(dT) was added to the washed streptavidin paramagnetic particles. After a 10 min incubation at room temperature, the paramagnetic particles along with the trapped mRNA were captured to the side of the tube

using a magnet. The supernatant was removed and the particles were washed four times with 0.1 X SSC (1.5 mL/wash). The mRNA was recovered by suspending the particles in 1.0 mL RNase-free water and removing the water while the particles were captured on the side of the tube. The water was placed, 500 μL at a time, into two 1.5 mL sterile microcentrifuge tubes. After the addition of 1/10th volume of 3 M sodium acetate (50 μL per tube), the mRNA was recovered by precipitation with an equal volume of isopropanol (550 μL per tube). The tubes were stored at -20°C overnight and then centrifuged at 14,000 rpm for 30 min at 4°C. The pellet was washed with 500 μL of 75% ice-cold ethanol and re-centrifuged. The ethanol was decanted and the pellet dried briefly under vacuum. The mRNA was dissolved in 60 μL of DEPC-treated nuclease-free sterile water. Quantitation was performed on 15 μL of the dissolved mRNA as described for total RNA. Approximately 9.6 μg of mRNA was recovered from 5 mg of total RNA.

l. Construction of cDNA library

First and second strand cDNA was synthesized using the ZAP-cDNA synthesis kit (Stratagene). Four μg of mRNA in 25 μL of water was incubated at 65°C for 5 min. Three μL of 100 mM methyl mercury was added and incubated at room temperature for 10 min. Four μL of 700 mM β-mercaptoethanol was added and incubation was continued for an additional 5 min. To the denatured mRNA 5 μL of 10x first strand buffer, 5 μL of 100 mM DTT, 3 μL nucleotide mixture (10 mM each dATP, dGTP, TTP and 5-methyl-dCTP), 2 μL of 1.4 μg/mL linker-primer, (5'GAGAGAGAGAGAGAGAGAGAACTAGTCTCGAGTTTTTTTTTTTTTTTTTT3'), 1 μL RNase block and 5 μL of water were added. The reaction was incubated at room temperature for 10 min to anneal the primer to the mRNA and 2.5 μL of 20 u/μL M-MuLV reverse transcriptase was added. Five μL of this reaction mixture was removed to a tube containing 0.5 μL of 800 Ci/mmole [a-^{32}P]dCTP (DuPont NEN). Both reactions were

incubated at 37°C for 1 hour. The radioactively labeled reaction was frozen at -20°C for later gel analysis.

To the 45 μL main reaction 40 μL of second strand buffer, 15 μL of 100 mM DTT, 6 μL of nucleotide mixture (10 mM dATP, dGTP, TTP and 26 mM dCTP), 268.3 μL water and 2 μL of 800 Ci/mmol [α-^{32}P]dCTP was added. After mixing, 4.5 μL of 1 u/μL RNase H and 19.2 μL of 5.2 u/μL *E. coli* DNA polymerase I were added and the reaction was incubated at 16°C for 2.5 hours. The reaction was extracted with 400 μL of phenol:chloroform (1:1) and the phases were separated by centrifugation. The aqueous phase was removed to a new tube and re-extracted with chloroform. The aqueous phase recovered as above. The double-stranded cDNA was recovered by precipitation overnight at -20°C after the addition of 33.3 μL of 3M sodium acetate and 867 μL of 100% ethanol. The precipitate was recovered by centrifugation in a microcentrifuge at 4°C for 60 min. The precipitate was washed with 1 μL of 80% ethanol and recovered by centrifugation at room temperature at full speed in a microcentrifuge. The supernatant was removed, the precipitate was dried under vacuum and dissolved in 45 μL of water. Three μL of the resuspended double-stranded cDNA was removed and frozen at -20°C until analyzed by gel electrophoresis.

To the remaining 42 μL of the double-stranded cDNA 5 μL of 10 x Klenow buffer (buffer #3), 2.5 μL of 2.5 mM nucleotides (dCTP, dGTP, dATP and TTP), and 0.5 μL of 5 u/μL Klenow fragment were added. After 30 min at 37°C, 50 μL of water were added and the reaction was extracted with an equal volume of phenol:chloroform (1:1) and then chloroform as described above. After the addition of 7 μL of 3M sodium acetate and 226 μL of 100% ethanol, the blunt-ended double-stranded DNA was recovered by precipitation by incubating on ice for 30 min and microcentrifuging at full speed at 4°C for 60 min. The pellet was washed with 300 μL of 80% ethanol, centrifuged and dried as before. Seven μL of 0.4 μg/μL *Eco*RI linkers were added to the dried cDNA. The structures of the *Eco*RI linkers are:

5' AATTCGGCACGAG 3'
3' GCCGTGCTC 5'

After vortexing to resuspend the cDNA, 1 μL of 10 x ligation buffer, 1 μL 10 mM ATP and 1 μL of 4 Weiss u/μL T4 DNA ligase was added and the reaction was incubated over night at 8°C. The ligase was inactivated by heating at 70°C for 30 min. The 5' ends of the *Eco*RI linkers attached to the cDNA were phosphorylated using polynucleotide kinase. One μL of 10x buffer #3, 2 μL of 10 mM ATP, 6 ML of water and 1 ML of 10 u/ML T4 polynucleotide kinase were added to the ligation reaction. After 30 min at 37°C the kinase reaction was heat inactivated at 70°C for 30 min.

*Xho*I "sticky ends" were generated at the end of the cDNA corresponding to the 3' end of the mRNA by digestion of the *Xho*I site in the linker-primer (see above). Twenty-eight μL of *Xho*I buffer and 3 μL of 40 u/mL *Xho*I were added to the cDNA and the reaction was incubated at 37°C for 1.5 hours. The cDNA with *Eco*RI sticky ends at the 5' end and *Xho*I sticky ends at the 3' end (relative to the original mRNA) were size fractionated by passage through a Sephacryl S-400 spin column as follows. Five μL of 10x STE (100mM tris (pH 7.0), 5 mM EDTA and 100 mM NaCl) was added and the cDNA was applied to the top of a 1 μL syringe containing Sephacryl S-400. A 500 ml microcentrifuge tube was placed on the bottom of the syringe and the column was placed in a centrifuge tube and centrifuged at about 400x*g* for 2 min. Sixty μL of 10x STE was added to the top of the syringe, a new microcentrifuge tube was placed on the bottom and the column was again centrifuged as before. This process was repeated until six fractions had been collected.

About 10% of each fraction was electrophoresed on a 1% agarose gel to determine the size distribution of the cDNA in each fraction. The remainder of each fraction was extracted with an equal volume of phenol:chloroform and then chloroform as described above and then precipitated by the addition of 2 volumes of 100% ethanol. After incubation at -20°C over night, the cDNA was recovered by centrifugation at 14,000 rpm at 4°C for 60 min in a microcentrofuge. The cDNA was washed with 200 μL of 80% ethanol as described above and dried. The cDNA was dissolved in 5 μL of water and 0.5 μL was removed to

determine the cDNA concentration by fluorography using the Hoefer TKO 100 DNA Fluorometer. The remaining 4.5 mL of fraction 1, containing the largest cDNA molecules, contained about 304 ng of cDNA.

One-hundred ng of cDNA from fraction 1 was ligated into 1 μg of Uni-Zap, a bacteriophage lambda ZAP vector that had been digested with *Eco*RI and *Xho*I (Stratagene). Fraction 1 cDNA (2.9 Ml) was added to 0.54 μL of 10 x ligation buffer, 0.5 μL 10 mM ATP, 1 μL of 1 μg/μL Uni-Zap XR vector and 0.5 μL of 4 Weiss u/μL T4 DNA ligase. The reaction was incubated at 8°C for about 44 hours. One μL aliquot of the ligation reaction was added to one aliquot of the 'Freeze-Thaw' extract from the Gigapack II Gold packaging kit (Stratagene). Fifteen μL of sonic extract was added and the contents were gently mixed. Packaging was carried out at room temperature. After 2 hours, 500 μL of SM buffer (0.01 M tris-HCL pH 7.5, 0.01 M $MgCl_2$ 0.1 mM Na_2EDTA) and 20 μL of chloroform was added to the packaging reaction, the debris was removed by a short centrifugation in a microcentrifuge and the packaged phages were stored at 4°C until used.

J. Titering of primary library.

One μL of the 500 μL primary library was mixed with 9 μL of SM buffer for a 1/10 dilution. One μL of this dilution was used to infect 200 μL of *E. coli* XL1-Blue MRF' cells grown to a density equal to an $O.D._{600}$= 0.5. The cells were incubated at 37°C for 15 min with gentle shaking. The infected cells were then mixed with 2.5 mL of 48°C top agar containing 15 μL of 0.5 M IPTG, and 50 μL of 250 mg/ml X-gal and plated on 100x15 mm NZY plates (5 g/L NaCl, 2 g/L $MgSO_4.7H_2O$, 5 g/L yeast extract, 10 g/L NZ amine [pH 7.5], and 15 g/L Difco agar). The plates were incubated overnight at 37°C. Background plaques were blue, while the recombinant plaques were white. The average of three such plates indicated that 1 μL of primary library produced 1,930 white recombinant plaques, and 65 blue plaques. The total 500 μL primary library was calculated to represent 965,000 recombinant plaques.

K. Amplification of primary library.

Into 20 sterile tubes 300 μL of *E. coli* XL1-Blue MRF' cells grown to an $O.D._{600}$= 0.5 were added. To each tube 12.5 μL of primary library stock, and 90 μL of SM buffer were added and the tubes were incubated at 37°C for 15 min. Two and one-half mL of 48°C top agar was added to each tube and the cells were plated on 100x15 mm NZY plates. The plates were incubated overnight at 37°C. Five mL of SM buffer were added to each plate and the plates were incubated for a further 8 hours at 4°C. The SM buffer was collected with a sterile pipette and stored in a sterile 250 mL centrifuge tube. Each plate was washed with about 4 mL of fresh SM buffer which was added to the previously collected material. Chloroform, to a final volume of 5%, was added to the amplified library. The library was then incubated at room temperature for 15 min and then centrifuged at 2,000x*g* for 10 min to remove cell debris. The supernatant (114.5 mL) was recovered and then transferred to a sterile polypropylene bottle. Chloroform was added to a final volume of 0.3% and the amplified library was stored at 4°C.

L. Titration of amplified library.

One μL of a 10^{-11} dilution of the amplified library in SM buffer contained 192 recombinant plaques when plated as described above. In order to obtain 50,000 recombinant plaques, 25 μL of a 10^{-7} dilution was used to infect 600 μL of *E. coli* XL1-Blue MRF' cells grown to an $O.D._{600}$= 0.5, which were then incubated at 37°C for 15 min. To these cells 6.5 mL of 48°C top agar was added and the library was plated on 150x15 mm NZY plates. Four such plates representing 200,000 recombinant plaques, were prepared and incubated at 37°C overnight. The plates were then chilled for 4 hours at 4°C, and then used for DNA screening of the library.

M. Polymerase Chain Reaction (PCR) amplification of xanthosine-N^7-methy transferase cDNA.

The synthesis of first strand cDNA was as described in the Stratagene protocol above. The two unique peptide sequences obtained by tryptic digestion allowed the synthesis of the degenerate primers depicted in Fig.4. A polymerase chain reaction (PCR) (Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. and Erlich, H.A., Science 239:487 (1988)) between pairs of these primers (1-6, 2-6, 3-5 or 4-5) using 4 ng cDNA, 1 μL 20 μM primers, 0.5 μL of each 1 mM deoxyribonucleotide triphosphate, 1.5 mM $MgCl_2$, 0.3 μL Taq DNA polymerase [5,000 u/mL], 2.5 μL 10x PCR buffer [10 mM tris-HCl (pH 9.0), 0.1% triton X-100] and sterile H_20 to a final volume of 25 μL was carried out. PCR conditions were 94°C for 4 min [1 cycle]; 94°C for 1 min, 43°C for 1 min, 72°C for 1 min [35 cycles]; 72°C for 5 min [1 cycle]). Reactions were done in 500 μL sterile microcentrifuge tubes using a Perkin Elmer DNA thermal cycler 480. Only the primer combination 1 and 6 resulted in a single product at an annealing temperature of 43°C. The product was measured by agarose gel electrophoresis using SeaPlaque agarose (FMC) to be approximately 750 base pairs. A commercially available 100 bp ladder was used as a size marker (Promega Corporation).

M. Cloning of coffee-specific xanthosine-N^7-methyltransferase PCR gene product.

The 750 bp fragment obtained using primers 1 and 6 (Fig.4) in a 50 μL PCR reaction had 50 μL of chloroform, and 100 μL of sterile water added to it. The mixture was vortexed and then centrifuged in a microcentrifuge at 14,000 rpm for 2 min. The top aqueous layer containing the DNA was removed and placed in a sterile tube. Ethidium-bromide plate quantitation indicated the presence of about 5 ng of about PCR amplfied DNA/μL. The PCR product was then ligated into a TA Cloning Kit pCR II vector (Invitrogen Corporation) in a 10 μL ligation reaction containing 1 μL 10 x ligation buffer, 2 μL pCR II vector (25 ng/μL), 3 μL fresh PCR product (5 ng/μL), 1 μL T4 DNA Ligase, and 3 μL of sterile water. The

ligation reaction was incubated at a 14°C overnight. The ligation reactions were centrifuged at 14,000 rpm for 2 min and placed on ice. To a freshly thawed vial of *E. coli* XL1-Blue competent cells 2 µL of 0.5 M â-mercaptoethanol was added and mixed gently with the pipette tip. Two µL of the ligation reaction was pipetted into the cells and they were stirred gently with the pipette tip to mix. The vial was then incubated on ice for 30 minutes and heat shocked for exactly 30 seconds in a 42°C heat-block. The vial was placed on ice. After 2 min 450 µL of sterile SOC medium (20 g/L tryptone, 5 g/L yeast extract, 0.5 g/L NaCl, 10 mL/L 250 mM KCl, 10 mL/L $MgCl_2$, 20 mL/L 1 M glucose, [pH 7.0]) was added to it. The vial was subsequently shaken at 225 rpm in a rotary shaker for 1 hour and then the placed on ice.

The transformed cells were plated by pipetting 50 µL and/or 200 µL from the cell suspension onto one of two LB plates (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 15 g/L Difco agar, pH 7.5) containing 50 µg/mL ampicillin and 40 µg/mL X-Gal. The plates were incubated at 37°C for 20 hours and then moved to 4°C for 3 hours to allow color development. Six white transformant colonies were analyzed for the presence and orientation of the PCR fragment.

N. Boiling plasmid mini-prep.

Each of the transformant colonies was grown in 5 mL sterile terrific broth (12 g/L tryptone, 24 g/L yeast extract, 4 mL/L glycerol, 100 mL/L 10x TB phosphate [0.17 M KH_2PO_4, 0.72 M K_2HPO_4]) supplemented with 50 µg/mL ampicillin. The tubes were incubated overnight in a rotary shaker at 37°C. Three mL of each colony was transferred to a 1.5 mL microcentrifuge tube, 1 mL at a time, and the cells concentrated by centrifugation at 14,000 rpm for 2 min. The supernatant was discarded each time and the cell pellet left as dry as possible. The cells were washed one time with 1 mL of sterile H_2O and centrifuged as before. The supernatant was discarded and the cell pellet resuspended in 320 µL STET buffer (8% sucrose, 0.5% triton X-100, 50 mM EDTA, 10 mM tris-HCl, pH 8.0). To these

cells, 32 μL of 10 mg/mL lysozyme in TE buffer (10 mL/L 1 M tris-HCl pH 8.0, 2 mL/L 0.5 M EDTA pH 8.0) was added and mixed by inverting the tubes several times. The tubes were placed in a boiling water bath for 5 min, and then placed immediately on ice. Once cooled they were centrifuged for 30 min at 14,000 rpm at 4°C. The pellet was removed from each tube with a sterile toothpick. The supernatant had 170 μL of 7.5 M NH_4OAc and 550 μL of ice-cold isopropanol added to it, and the DNA was precipatated overnight at -20°C. The tubes were centrifuged at 14,000 rpm at 4°C for 30 min, and the pellet washed with 75% ethanol and dried for 1 min in a speed-vac. The DNA was resuspended in 50 μL of sterile H_2O containing 1 μL of 5 mg/mL RNase A.

O. Restriction digestion to remove insert from pCR II plasmid.

A reaction mixture of 25 μL was prepared by adding 15 μL of plasmid mini-prep DNA as obtained above, 2.5 μL of buffer H (90 mM tris-HCl [pH 7.5], 10 mM $MgCl_2$, 50 mM NaCl), 1 μL of *Eco*RI (8-12 u/μL), and 6.5 μL of sterile H_2O. The mixture was incubated in a shaking water bath at 37°C for 1 hour, and then boiled in a water bath for 1 min. The tubes were centrifuged at 14,000 rpm for 15 seconds and then allowed to cool down to room temperature. To 10 μL of each mixture 2 μL of loading dye was added, and the digestion products were analyzed by 1.5% agarose gel electrophoresis using ultra-pure agarose (GibcoBRL) and a 100 bp ladder as a size marker (Promega Corporation).

Only one of the six reactions indicated the presence of a digested insert of ~750 bp. The original bacterial colony corresponding to the plasmid with the 750bp xanthosine-N^7-methyl transferase PCR product was inoculated into a 250 mL Erlenmeyer flask containing 50 mL of sterile LB media (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.5) supplemented with 50 μg/mL ampicillin. The flask was incubated in a rotary shaker at 30°C overnight. In a 1.5 mL microcentrifuge tube 18 mL of the resulting cell media was concentrated by centrifugation as above.

Plasmid DNA was purified using the QIAGEN plasmid mini kit procedure (Qiagen Inc.). The washed bacterial pellet was resuspended in 0.3 mL of buffer P1 which contains the supplied RNase. To this 0.3 mL of alkaline lysis buffer P2 was added, mixed gently by flicking the tube and incubated for no longer than 5 min at room temperature. Next 0.3 mL of chilled buffer P3 was added and mixed by inverting the tube 6 times. After 10 min on ice the extract was centrifuged 14,000 rpm for 15 min in a microcentrifuge. The supernatant was removed and applied to a QIAGEN-tip 20 that was previously equilibrated by the application of 1 mL QBT buffer by gravity flow. The applied cell extract supernatant was also allowed to enter the resin of the column by gravity flow. Once the flow through the column had stopped, the QIAGEN-tip 20 was washed 4 times with one mL buffer QC. The DNA was eluted by washing the QIAGEN-tip 20 with 0.8 mL buffer QF and precipitated by the addition of 0.7 volumes (560 μL) of room temperature isopropanol. The tube was immediately centrifuged at 14,000 rpm for 30 min and the supernatant carefully removed. The precipitated DNA was washed with 1 mL of ice-cold 70% ethanol, centrifuged as above, and air dried for 5 min. The DNA was resuspended in 100 μL of sterile H_2O. UV spectrophotometry, as described above, on 1 μL of the DNA resuspension indicated that there was 55 μg of purified recombinant pCRII plasmid DNA per 100 μL.

Automated DNA sequencing of the insert in the pCRII plasmid from its 5' end was accomplished using the M13 reverse primer which binds to a reference in pCRII just adjacent to the site where the PCR product was inserted. Sequencing was done at the University of Hawaii Biotechnology service facility. The sequencing reaction contained 1 μg of plasmid template and 3.2 pmol M13 primer. The sequence obtained indicated that the PCR product coded for the DNA sequence of the first 6 amino acids of peptide fragments A and B (Fig.4) from whose sequence the degenerate DNA primers 1 and 2 (Fig.4) were made. In addition, the sequence also coded for the following 7 amino acids of the peptide fragment, the DNA sequence of which was not used in primer construction. So in effect the DNA sequence for the correct protein was cloned.

P. Making of a random primed probe for cDNA screening using the PCR product.

Two 25μL restriction digestions with *Eco*R1 were carried out on two 17.5 μL aliquots of the purified pCRII plasmid as described above. The products were separated on a 1% agarose gel as before, and the 750 bp insert was excised aseptically from two lanes of the gel. The gel pieces having a mass of 0.65 g were transferred into a sterile 40 mL polypropylene tube and subjected to Geneclean II kit purification (BIO 101, Inc). Four and one-half volumes of NaI (2.93 mL) stock solution was added to the gel slices. One-half the volume of the gel TBE modifier (325μL) was added and the tube incubated at 45°C for 5 min. To this 15 μL of glassmilk suspension was added and incubated for a further 5 min. The glassmilk/DNA complex was pelleted by centrifugation for 10 sec at 1,000 rpm and the supernatant was removed. The glassmilk pellet was washed 3 times with 1 mL New Wash solution and the DNA was eluted with 50 μL of sterile H_2O. Ethidium bromide plates indicated that the DNA concentration was 10 ng/μL.

A random primed probe was systhesized from 30 ng (3μL) of the purified DNA. Three μL of the DNA was added to 27 μL of sterile water and the DNA was denatured by heating in a boiling water bath. To this the Promega Corporations Prime-a-Gene kit constituents (10 μL 5x labeling buffer, 2 μL of unlabeled dNTP's [20 μM each dCTP, dGTP, TTP], 2 μL 1 mg/mL acetylated BSA, 1 μL 5u/μL Klenow enzyme) and 5 μL of [α-^{32}P]dATP (50 μCi, 3,000 Ci/mmole; DuPont NEN) were added to a final volume of 50 μL, and allowed to incubate at room temperature for 1 hour. The reaction was terminated by the addition of 2 μL 0.5 M Na_2EDTA (20 mM final concentration) and heated for 2 min in a boiling water bath.

Q. Screening of amplified library with random primed probe.

The four 150x15 mm NZY plates that had approximately 50,000 recombinant clones per plate were chilled to 4°C (see above for plating and growth conditions), and the

recombinant plaques lifted by first presoaking 132 mm Magna nylon transfer membranes (MSI Corporation) on chromatography paper saturated with 5x SSC buffer for 10 sec. The membranes were placed onto the plates containing the recombinant plaques for 5 min, and then lifted and placed, phage containing side up, for 2 min on chromatography paper saturated with 0.5 M NaOH and 1.5 M NaCl. The membranes were neutralized by transferring onto chromatography paper saturated with 0.5 M tris-HCl (pH 8.0) and 1.5 M NaCl for 5 min. They were then placed for 20 sec on chromatography paper saturated with 2x SCC buffer, 0.2 M tris-HCL (pH 7.5) and then blotted dry. After 1 hour of air drying, the DNA was cross-linked to the membranes by exposure to 12,000 μJoules of UV using a UV Stratalinker 1800 (Stratagene Corporation). The four membranes were prehybridized at 65°C for 2 hours in 100 mL 6x SSPE (52.2 g/L NaCl, 8.3 g/L $NaH_2PO_4.H_2O$, 2.2 g/L Na_2EDTA, [pH 7.4]), 5x Denhardt's solution (1 g/L Ficoll, 1 g/L polyvinylpyrrolidone, 1 g/L BSA [pentax fraction V]), 0.5% SDS and 100 μg/mL denatured herring sperm DNA in a Hybrid Mark II hybridization oven.

Hybridization was carried out at 65°C for 12 hours in 10 mL of 6x SSPE, 0.5% SDS, 100 μg/mL powdered/denatured herring sperm DNA, and 52 μL 15 X10^6 dpms/ml of the random primed probe described above. At the end of the hybridization period the probe was removed and the membranes briefly washed for 30 sec with 100 mL of 65°C 2x SSC containing 0.5% SDS. The membranes were then washed for an additional 30 min with the same amount and concentration of fresh buffer. The membranes were subjected to two more 100 mL washes for 30 min with 65°C, 0.2x SSC, 0.5% SDS, and then rapped in a cellophane envelope and exposed to pre-flashed Fuji RX_{GCU} X-ray film at -70°C for 24 hours. Fifteen positive clones were observed. These plaques were picked and placed in 1 mL SM buffer containing 20 μL chloroform (phage stock). Of these, 11 were processed to secondary or tertiary screening until single individual plaques were obtained.

R. Characterization of xanthosine-N^7-methyltransferase cDNA clones.

The sizes of the putative xanthosine-N^7-methyltransferase cDNA clones were determined by polymerase chain reaction using primers homologous to the T3 and T7 promoters that are present in the cloning vector and that flank the cDNA insertion site. Conditions for polymerase chain reaction were as described above except that the cycle was 35 cycles of 95°C for 1 minute, 50°C for 1 minute and 72°C for 2 minutes. Analysis was by agarose gel electrophoresis as before. The three largest clones obtained were subjected to *in vivo* excision by mixing in a sterile tube 200 µL of single plaque phage stock with 200 µL of fresh XL1-Blue MRF' cells grown to an $O.D._{600}$=1.0. To this mixture 1 µL of ExAssist (Stratagene Corporation) helper phage (>1×10^6 pfu/µL) was added and the tubes were incubated at 37°C for 15 min. Three mL of sterile LB broth was added and incubation was continued for 3 hours at 37°C with shaking. The cultures were heated in a 70°C water bath for 20 min, and then the tubes centrifuged at 1,000x*g* for 15 min. One mL of the supernatant containing the excised pBluesript phagemid packaged as a filamentous phage particle was transferred to a sterile 1.5 mL microcentrifuge tube and stored at 4°C as the stock solution. Twenty-five µL of the stock solution was added to 200 µL of *E. coli* Solar cells grown to an $O.D._{600}$=1 in a microcentrifuge tube. After incubation at 37°C for 15 min, the 200 µL cells were plated on 100x15 mm NZY agar plates containing 50 µg/mL ampicillin. The plates were incubated overnight at 37°C until colonies appeared. A single colony was inoculated into 10 mL of sterile LB broth containing 50 µg/mL ampicillin and grown overnight at 37°C with shaking. The 10 mL of cell culture was concentrated in a 1.5 mL sterile microcentrifuge tube and the pelleted cells subjected to QIAGEN plasmid purification as described previously. The purified plasmid DNA was resuspended in 50 µL of sterile H_2O. DNA automated sequencing reactions were performed by mixing 8 µL of this DNA sample (0.8 µg) with 4 µL of either T3 or T7 sequencing primers (0.8 pmol/µL). The remainder of the process was as previously described. Each sequencing reaction gave aproximately 350 bases of sequence. The sequence is shown in Figure 5. Thre amino acid sequence of xanthosine-

N^7-methyl transferase as predicted from the base sequence of the cDNA is shown in Figure 6.

The foregoing examples are for illustrative purposes only, and should not be viewed as limiting the scope of applicants' invention, which is set forth in the claims appended hereto.

CLAIMS

WHAT IS CLAIMED IS:

1. Substantially pure xanthosine N^7 methyl transferase, characterized by:

a) methylation of xanthosine at the 7 position of the purine ring, using S-adenosylmethionine as substrate;

b) comprising tryptic fragments with the amino acid sequences

(1) Ile, Asn, Tyr, Ala, Ser, Gly, Ala, Ser, Gly, Ile, Leu, Asp, Gln, Thr; and

(2) Gly, Tyr, Val, Pro, Cys, Tur, Phe, (Thr/Asp) Phe, Ile, Asp, Asp, Gln, Asp.

2. A substantially pure xanthosine-N^7-methyltransferase consisting essentially of the amino acid sequence:

1Met Ala Phe Val Ala Arg Gln Trp Phe Leu Leu Ser Ile

14Ile Asn Val Val Val Val Cys Phe Leu Lys Pro Phe Ala

27Leu Gly Glu Gln Gln Val Pro Cys Tyr Phe Ile Phe Gly

40Asp Ser Gln Asp Asp Asn Gly Asn Asn Asn His Leu Asn

53Thr Thr Ala Arg Ala Asn Tyr Pro Pro Tyr Gly Ile Asp

66Phe Pro Glu Gly Pro Thr Gly Arg Phe Thr Asn Gly Arg

79Asn His Ala Asp Phe Ile Gly Glu Leu Leu Gly Phe Asp

92Ser Tyr Ile Pro Pro Phe Ala Asn Thr Lys Gly Arg Asp

105Ile Thr Lys Gly Ile Asn Tyr Ala Ser Gly Ala Ser Gly

118Ile Leu Asp Gln Thr Gly Arg His Leu Gly Asp Leu Phe

131Ser Phe Asn Glu Gln Leu His Asn His Glu Arg Ala Ile

144Ser Arg Ile Val Arg Leu Ile Gly Asn Arg Ser Ala Thr

157Lys Glu Tyr Leu Ala Lys Cys Leu Tyr Thr Val Ala Leu

170Gly Asn Asn Asp Tyr Ile Asn Asn Tyr Leu Leu Pro Glu

183Tyr Tyr Pro Thr Ser His Leu Tyr Thr Pro Arg Glu Phe

196Ala Ser Leu Leu Ile Arg His Tyr Ser Gln Gln Leu Arg

209Thr Leu Tyr Arg Leu Gly Ala Arg Lys Ile Ala Val Phe

222Gly Leu Gly Trp Leu Gly Cys Ile Pro Ala Glu Leu Ser

235Thr Asp Gly Asn Cys Val Asp Ser Ile AsnGlu Glu Val

248Leu Leu Phe Asn Asp Lys LeuLys Pro Leu Val Asp Glu

261Leu Asn Thr Glu Leu Ser Gly Ala Gln Phe Leu Tyr Val

274Asp Val Ile Ala Ile Asn Leu Asn Asn Leu Ser Thr Pro

287Ala Glu Ile Thr Ile Gly Asn Ala Pro Cys Cys Asn Val

300Ser Ala Ala Val Ala Gly Gly Gln Cys Ile Pro Gly Gln

313Ile Pro Cys Ser Asn Arg Asn Gln Tyr Tyr Phe Trp Asp

326Asp Phe His Pro Ser Glu Val Val Asn Glu Ala Tyr Ser

339Arg Leu Ala Tyr Ser Ala Leu Ser Ser Leu Leu AspAla

352Asp Pro Leu Ala Ile Gly Gly Leu Thr Gly Lys Asn Cys

365His Asp Lys Val Lys Ile Gln.

3. Substantially pure nucleic acid sequence that codes on expression for xanthosine N^7 methyltransferase comprising:

a) 1 CCTCTGACTT GCTAAACCTA CCATTACCTT TTTCTTCTTG TCATCTGCAT

51 TCATGGCTTT TGTAGCCAGG CAATGGTTTC TCCTATCCAT CATTAATGTA

101 GTGGTTGTCT GTTTCTTGAA ACCATTTGCC CTAGGCGAAC AACAGGTCCC

151 TTGCTACTTC ATTTTTGGAG ACTCACAAGA TGACAATGGC AACAATAATC

201 ACCTGAACAC CACTGCCAGG GCAAATTATC CACCTTACGG CATTGATTTC

251 CCAGAAGGTC CAACTGGTCG CTTCACCAAT GGTCGAAATC ATGCAGACTT

301 CATTGGTGAG CTCCTTGGAT TTGACAGCTA CATACCTCCA TTTGCAAATA

351 CAAAAGGCCG GGATATCACT AAAGGCATTA ATTATGCTTC GGGAGCATCT

401 GGAATTCTTG ATCAGACCGG TCGTCACCTG GGCGATCTCT TCAGCTTCAA

451 CGAACAATTG CACAATCACG AGAGAGCAAT TTCGCGCATC GTGCGGTTGA

501 TTGGAAACAG ATCTGCAACA AAAGAATATC TAGCCAAATG TCTGTACACT

551 GTTGCATTGG GGAATAATGA TTACATCAAC AACTACTTGT TGCCAGAATA

601 TTATCCTACC AGCCACCTAT ATACTCCAAG AGAATTTGCC AGCTTGTTAA

651 TTAGGCATTA TTCTCAGCAA CTACGGACTT TGTACAGATT GGGGGCAAGA

701 AAAATAGCCG TTTTTGGGCT TGGTTGGCTT GGCTGCATAC CTGCTGAGTT

751 ATCTACAGAT GGTAACTGTG TGGATTCTAT TAACGAGGAA GTTCTGTTAT

801 TCAATGACAA GCTCAAGCCA CTGGTTGATG AACTGAATAC CGAGTTAAGC

851 GGTGCACAAT TTCTTTATGT AGATGTGATA GCAATCAATT TGAACAATTT

901 ATCCACCCCT GCAGAAATTA CAATTGGCAA TGCACCATGC TGCAACGTGT

951 CTGCAGCAGT TGCTGGTGGA CAGTGTATTC CTGGGCAAAT TCCCTGCAGC

1001 AACAGGAACC AATATTATTT TTGGGATGAT TTCCATCCCA GTGAAGTAGT

1051 CAATGAAGCA TATTCAAGAT TAGCATATTC TGCGTTATCC TCATTACTTG

1101 ATGCTGATCC TCTTGCCATT GGCGGCCTAA CAGGCAAAAA CTGTCATGAT

1151 AAAGTGAAGA TACAATAGAC TGTATCTATG TGTCCCATGA TATTTCTATA

1201 TTCCAAGTTT CCGACAAGTC AAACTCAATG TAATAAAACT TGAGAGTCCG

1251 AATGTGCTAG TGTGATGTTA TCTCCTCAAT GGAAACAATA TGTTATCATT

1301 AATCTCAGAC TATTTATAAT TACTATTAAA AAAAAAAAAA AAAAAAA;

b) DNA sequences which hybridize to the foregoing DNA sequence, said hybridizing sequences consisting essentially of sequences that are complementary to the foregoing DNA sequence; and

c) DNA sequences that are degenerate with respect to the foregoing DNA sequences.

4. A method for producing caffeine free coffee beans comprising:

a) transforming coffee plants with a DNA sequence that is antisense to the DNA sequence:

```
  1 CCTCTGACTT GCTAAACCTA CCATTACCTT TTTCTTCTTG TCATCTGCAT
 51 TCATGGCTTT TGTAGCCAGG CAATGGTTTC TCCTATCCAT CATTAATGTA
101 GTGGTTGTCT GTTTCTTGAA ACCATTTGCC CTAGGCGAAC AACAGGTCCC
151 TTGCTACTTC ATTTTTGGAG ACTCACAAGA TGACAATGGC AACAATAATC
201 ACCTGAACAC CACTGCCAGG GCAAATTATC CACCTTACGG CATTGATTTC
251 CCAGAAGGTC CAACTGGTCG CTTCACCAAT GGTCGAAATC ATGCAGACTT
301 CATTGGTGAG CTCCTTGGAT TTGACAGCTA CATACCTCCA TTTGCAAATA
351 CAAAAGGCCG GGATATCACT AAAGGCATTA ATTATGCTTC GGGAGCATCT
401 GGAATTCTTG ATCAGACCGG TCGTCACCTG GGCGATCTCT TCAGCTTCAA
451 CGAACAATTG CACAATCACG AGAGAGCAAT TTCGCGCATC GTGCGGTTGA
501 TTGGAAACAG ATCTGCAACA AAAGAATATC TAGCCAAATG TCTGTACACT
551 GTTGCATTGG GGAATAATGA TTACATCAAC AACTACTTGT TGCCAGAATA
601 TTATCCTACC AGCCACCTAT ATACTCCAAG AGAATTTGCC AGCTTGTTAA
651 TTAGGCATTA TTCTCAGCAA CTACGGACTT TGTACAGATT GGGGGCAAGA
701 AAAATAGCCG TTTTTGGGCT TGGTTGGCTT GGCTGCATAC CTGCTGAGTT
751 ATCTACAGAT GGTAACTGTG TGGATTCTAT TAACGAGGAA GTTCTGTTAT
801 TCAATGACAA GCTCAAGCCA CTGGTTGATG AACTGAATAC CGAGTTAAGC
851 GGTGCACAAT TTCTTTATGT AGATGTGATA GCAATCAATT TGAACAATTT
```

901 ATCCACCCCT GCAGAAATTA CAATTGGCAA TGCACCATGC TGCAACGTGT

951 CTGCAGCAGT TGCTGGTGGA CAGTGTATTC CTGGGCAAAT TCCCTGCAGC

1001 AACAGGAACC AATATTATTT TTGGGATGAT TTCCATCCCA GTGAAGTAGT

1051 CAATGAAGCA TATTCAAGAT TAGCATATTC TGCGTTATCC TCATTACTTG

1101 ATGCTGATCC TCTTGCCATT GGCGGCCTAA CAGGCAAAAA CTGTCATGAT

1151 AAAGTGAAGA TACAATAGAC TGTATCTATG TGTCCCATGA TATTTCTATA

1201 TTCCAAGTTT CCGACAAGTC AAACTCAATG TAATAAAACT TGAGAGTCCG

1251 AATGTGCTAG TGTGATGTTA TCTCCTCAAT GGAAACAATA TGTTATCATT

1301 AATCTCAGAC TATTTATAAT TACTATTAAA AAAAAAAAAA AAAAAAA ; and

b) harvesting the fruit from the transformed coffee plants.

5. A method for producing caffeine free coffee beans comprising: transforming coffee plants with a DNA sequence that is antisense to the DNA sequence that codes on expression for:

1 Met Ala Phe Val Ala Arg Gln Trp Phe Leu Leu Ser Ile

14 Ile Asn Val Val Val Val Cys Phe Leu Lys Pro Phe Ala

27 Leu Gly Glu Gln Gln Val Pro Cys Tyr Phe Ile Phe Gly

40 Asp Ser Gln Asp Asp Asn Gly Asn Asn Asn His Leu Asn

53 Thr Thr Ala Arg Ala Asn Tyr Pro Pro Tyr Gly Ile Asp

66 Phe Pro Glu Gly Pro Thr Gly Arg Phe Thr Asn Gly Arg

79 Asn His Ala Asp Phe Ile Gly Glu Leu Leu Gly Phe Asp

92 Ser Tyr Ile Pro Pro Phe Ala Asn Thr Lys Gly Arg Asp

105 Ile Thr Lys Gly Ile Asn Tyr Ala Ser Gly Ala Ser Gly

118 Ile Leu Asp Gln Thr Gly Arg His Leu Gly Asp Leu Phe

131 Ser Phe Asn Glu Gln Leu His Asn His Glu Arg Ala Ile

144 Ser Arg Ile Val Arg Leu Ile Gly Asn Arg Ser Ala Thr
157 Lys Glu Tyr Leu Ala Lys Cys Leu Tyr Thr Val Ala Leu
170 Gly Asn Asn Asp Tyr Ile Asn Asn Tyr Leu Leu Pro Glu
183 Tyr Tyr Pro Thr Ser His Leu Tyr Thr Pro Arg Glu Phe
196 Ala Ser Leu Leu Ile Arg His Tyr Ser Gln Gln Leu Arg
209 Thr Leu Tyr Arg Leu Gly Ala Arg Lys Ile Ala Val Phe
222 Gly Leu Gly Trp Leu Gly Cys Ile Pro Ala Glu Leu Ser
235 Thr Asp Gly Asn Cys Val Asp Ser Ile Asn Glu Glu Val
248 Leu Leu Phe Asn Asp Lys Leu Lys Pro Leu Val Asp Glu
261 Leu Asn Thr Glu Leu Ser Gly Ala Gln Phe Leu Tyr Val
274 Asp Val Ile Ala Ile Asn Leu Asn Asn Leu Ser Thr Pro
287 Ala Glu Ile Thr Ile Gly Asn Ala Pro Cys Cys Asn Val
300 Ser Ala Ala Val Ala Gly Gly Gln Cys Ile Pro Gly Gln
313 Ile Pro Cys Ser Asn Arg Asn Gln Tyr Tyr Phe Trp Asp
326 Asp Phe His Pro Ser Glu Val Val Asn Glu Ala Tyr Ser
339 Arg Leu Ala Tyr Ser Ala Leu Ser Ser Leu Leu Asp Ala
352 Asp Pro Leu Ala Ile Gly Gly Leu Thr Gly Lys Asn Cys
365 His Asp Lys Val Lys Ile Gln.

Figure 1. Caffeine Biosynthetic Pathway

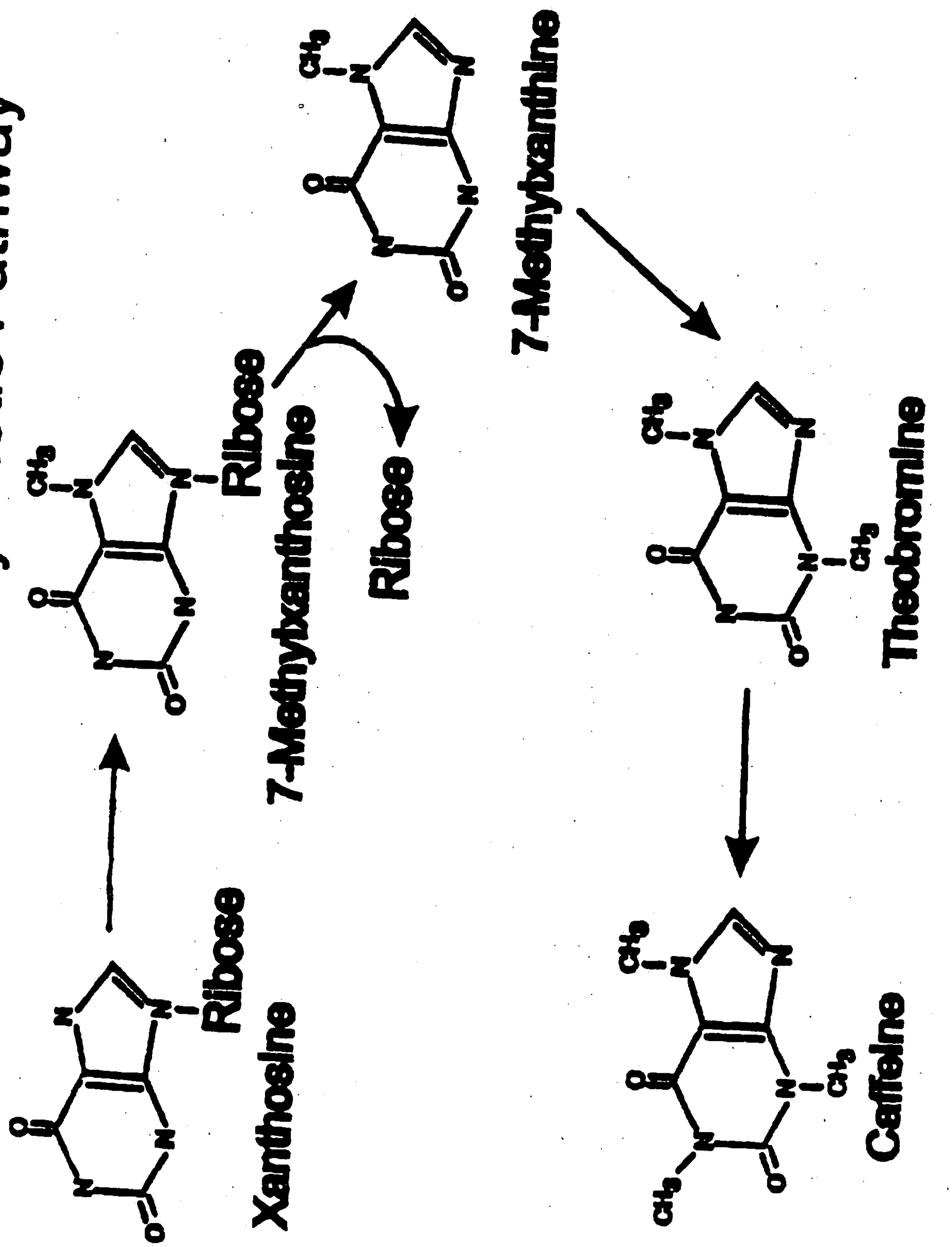

Figure 2 Polyacrylamide gel of purified xanthosine-N7-methyltransferase

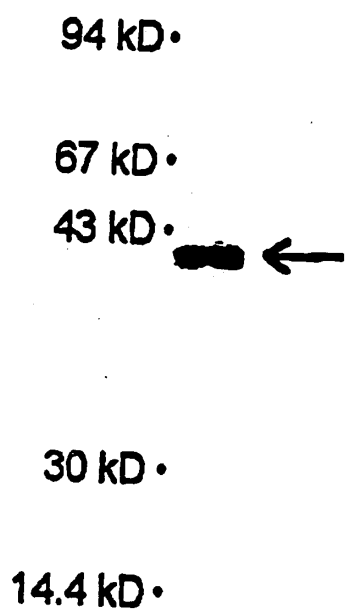

Figure 3 HPLC separation of tryptic digest of xanthosine-N7-methyl transferase

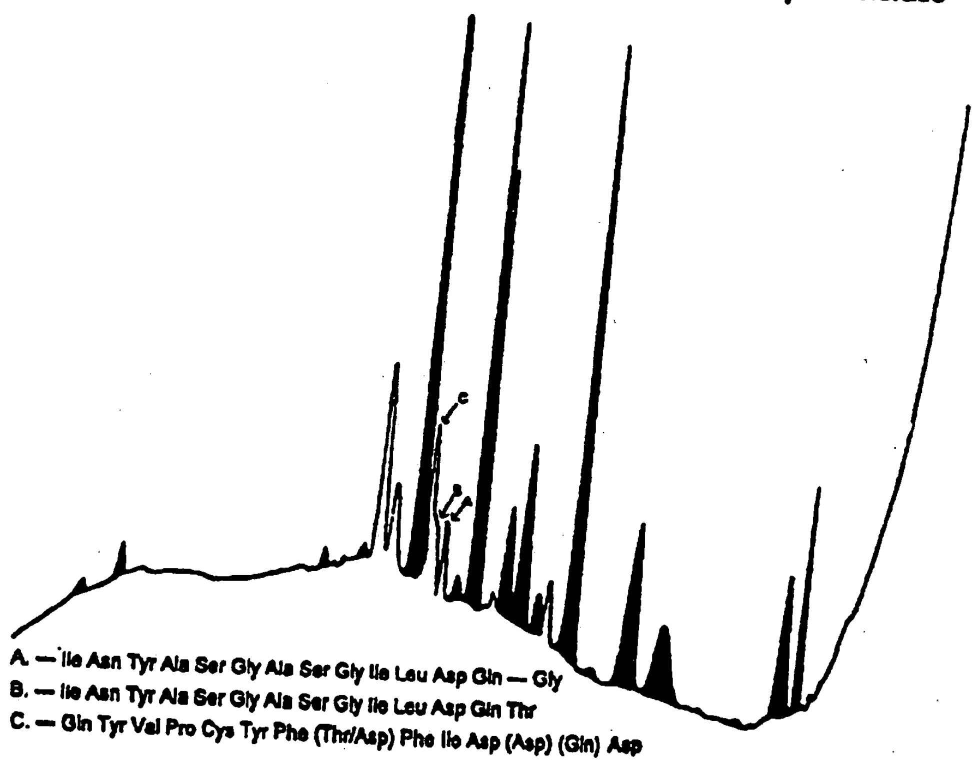

Figure 4 Oligonucleotides synthesized from peptides

Fragment A.	--- Ile Asn Tyr Ala Ser Gly Ala Ser Gly Ile Leu Asp Gln — Gly
Fragment B.	— Ile Asn Tyr Ala Ser Gly Ala Ser Gly Ile Leu Asp Gln Thr
Primer 1	5'ATI AA$^{C}_{T}$ TA$^{C}_{T}$GCI TCI GGI GC
Primer 2	5'ATI AA$^{C}_{T}$ TA$^{C}_{T}$GCI AG$^{C}_{T}$ GGI GC
Primer 3	3'TAI TT$^{G}_{A}$AT$^{G}_{A}$CGI AGI CCI GC
Primer 4	3'TAI TT$^{G}_{A}$AT$^{G}_{A}$CGI TC$^{G}_{A}$ CCI GC

Fragment C.	— Gln Tyr Val Pro Cys Tyr Phe $^{Thr}_{Asp}$ Phe Ile Asp (Asp) (Gln) Asp
Primer 5	5'CA$^{G}_{A}$TAT GTI CCI TGT TAT TT
Primer 6	3'GTT AT$^{G}_{A}$CAI GGI AC$^{G}_{A}$ AT$^{G}_{A}$AA

FIGURE 5

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   1 CCTCTGACTT GCTAAACCTA CCATTACCTT TTTCTTCTTG TCATCTGCAT
  51 TCATGGCTTT TGTAGCCAGG CAATGGTTTC TCCTATCCAT CATTAATGTA
 101 GTGGTTGTCT GTTTCTTGAA ACCATTTGCC CTAGGCGAAC AACAGGTCCC
 151 TTGCTACTTC ATTTTTGGAG ACTCACAAGA TGACAATGGC AACAATAATC
 201 ACCTGAACAC CACTGCCAGG GCAAATTATC CACCTTACGG CATTGATTTC
 251 CCAGAAGGTC CAACTGGTCG CTTCACCAAT GGTCGAAATC ATGCAGACTT
 301 CATTGGTGAG CTCCTTGGAT TTGACAGCTA CATACCTCCA TTTGCAAATA
 351 CAAAAGGCCG GGATATCACT AAAGGCATTA ATTATGCTTC GGGAGCATCT
 401 GGAATTCTTG ATCAGACCGG TCGTCACCTG GGCGATCTCT TCAGCTTCAA
 451 CGAACAATTG CACAATCACG AGAGAGCAAT TTCGCGCATC GTGCGGTTGA
 501 TTGGAAACAG ATCTGCAACA AAAGAATATC TAGCCAAATG TCTGTACACT
 551 GTTGCATTGG GGAATAATGA TTACATCAAC AACTACTTGT TGCCAGAATA
 601 TTATCCTACC AGCCACCTAT ATACTCCAAG AGAATTTGCC AGCTTGTTAA
 651 TTAGGCATTA TTCTCAGCAA CTACGGACTT TGTACAGATT GGGGGCAAGA
 701 AAAATAGCCG TTTTTGGGCT TGGTTGGCTT GGCTGCATAC CTGCTGAGTT
 751 ATCTACAGAT GGTAACTGTG TGGATTCTAT TAACGAGGAA GTTCTGTTAT
 801 TCAATGACAA GCTCAAGCCA CTGGTTGATG AACTGAATAC CGAGTTAAGC
 851 GGTGCACAAT TTCTTTATGT AGATGTGATA GCAATCAATT TGAACAATTT
 901 ATCCACCCCT GCAGAAATTA CAATTGGCAA TGCACCATGC TGCAACGTGT
 951 CTGCAGCAGT TGCTGGTGGA CAGTGTATTC CTGGGCAAAT TCCCTGCAGC
1001 AACAGGAACC AATATTATTT TTGGGATGAT TTCCATCCCA GTGAAGTAGT
1051 CAATGAAGCA TATTCAAGAT TAGCATATTC TGCGTTATCC TCATTACTTG
1101 ATGCTGATCC TCTTGCCATT GGCGGCCTAA CAGGCAAAAA CTGTCATGAT
1151 AAAGTGAAGA TACAATAGAC TGTATCTATG TGTCCCATGA TATTTCTATA
1201 TTCCAAGTTT CCGACAAGTC AAACTCAATG TAATAAAACT TGAGAGTCCG
1251 AATGTGCTAG TGTGATGTTA TCTCCTCAAT GGAAACAATA TGTTATCATT
1301 AATCTCAGAC TATTTATAAT TACTATTAAA AAAAAAAAAA AAAAAAA
```

FIGURE 6

1	Met	Ala	Phe	Val	Ala	Arg	Gln	Trp	Phe	Leu	Leu	Ser	Il
14	Ile	Asn	Val	Val	Val	Val	Cys	Phe	Leu	Lys	Pro	Phe	Ala
27	Leu	Gly	Glu	Gln	Gln	Val	Pro	Cys	Tyr	Phe	Ile	Phe	Gly
40	Asp	Ser	Gln	Asp	Asp	Asn	Gly	Asn	Asn	Asn	His	Leu	Asn
53	Thr	Thr	Ala	Arg	Ala	Asn	Tyr	Pro	Pro	Tyr	Gly	Ile	Asp
66	Phe	Pro	Glu	Gly	Pro	Thr	Gly	Arg	Phe	Thr	Asn	Gly	Arg
79	Asn	His	Ala	Asp	Phe	Ile	Gly	Glu	Leu	Leu	Gly	Phe	Asp
92	Ser	Tyr	Ile	Pro	Pro	Phe	Ala	Asn	Thr	Lys	Gly	Arg	Asp
105	Ile	Thr	Lys	Gly	Ile	Asn	Tyr	Ala	Ser	Gly	Ala	Ser	Gly
118	Ile	Leu	Asp	Gln	Thr	Gly	Arg	His	Leu	Gly	Asp	Leu	Phe
131	Ser	Phe	Asn	Glu	Gln	Leu	His	Asn	His	Glu	Arg	Ala	Ile
144	Ser	Arg	Ile	Val	Arg	Leu	Ile	Gly	Asn	Arg	Ser	Ala	Thr
157	Lys	Glu	Tyr	Leu	Ala	Lys	Cys	Leu	Tyr	Thr	Val	Ala	Leu
170	Gly	Asn	Asn	Asp	Tyr	Ile	Asn	Asn	Tyr	Leu	Leu	Pro	Glu
183	Tyr	Tyr	Pro	Thr	Ser	His	Leu	Tyr	Thr	Pro	Arg	Glu	Phe
196	Ala	Ser	Leu	Leu	Ile	Arg	His	Tyr	Ser	Gln	Gln	Leu	Arg
209	Thr	Leu	Tyr	Arg	Leu	Gly	Ala	Arg	Lys	Ile	Ala	Val	Phe
222	Gly	Leu	Gly	Trp	Leu	Gly	Cys	Ile	Pro	Ala	Glu	Leu	Ser
235	Thr	Asp	Gly	Asn	Cys	Val	Asp	Ser	Ile	Asn	Glu	Glu	Val
248	Leu	Leu	Phe	Asn	Asp	Lys	Leu	Lys	Pro	Leu	Val	Asp	Glu
261	Leu	Asn	Thr	Glu	Leu	Ser	Gly	Ala	Gln	Phe	Leu	Tyr	Val
274	Asp	Val	Ile	Ala	Ile	Asn	Leu	Asn	Asn	Leu	Ser	Thr	Pro
287	Ala	Glu	Ile	Thr	Ile	Gly	Asn	Ala	Pro	Cys	Cys	Asn	Val
300	Ser	Ala	Ala	Val	Ala	Gly	Gly	Gln	Cys	Ile	Pro	Gly	Gln
313	Ile	Pro	Cys	Ser	Asn	Arg	Asn	Gln	Tyr	Tyr	Phe	Trp	Asp
326	Asp	Phe	His	Pro	Ser	Glu	Val	Val	Asn	Glu	Ala	Tyr	Ser
339	Arg	Leu	Ala	Tyr	Ser	Ala	Leu	Ser	Ser	Leu	Leu	Asp	Ala
352	Asp	Pro	Leu	Ala	Ile	Gly	Gly	Leu	Thr	Gly	Lys	Asn	Cys
365	His	Asp	Lys	Val	Lys	Ile	Gln						

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/04982

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :530/350; 435/69.1, 172.3, 193, 320.1; 536/23.6, 24.1; 800/205
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350; 435/69.1, 172.3, 193, 320.1; 536/23.6, 24.1; 800/205

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CABA, CAPLUS, BIOSIS, MEDLINE
search terms: caffeine, xanthosine n7 methyl transferase, recombinant, plant, antisense

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,334,529 A (ADAMS et al) 02 August 1994, columns 1-12.	1-5
Y	SCHULTHESS et al. Are xanthosine and 7-methylxanthosine caffeine precursors? Phytochemistry. 1995, Vol. 39, No. 6, pages 1363-1370.	1-5
Y	HOFGEN et al. A visible marker for antisense mRNA expression in plants: Inhibition of chlorophyll synthesis with a glutamate-1-semialdehyde aminotransferase antisense gene. Proc. Natl. Acad. Sci. USA. March 1994, Vol. 91, pages 1726-1730.	1-5

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
17 JULY 1997

Date of mailing of the international search report
15 AUG 1997

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
IW for
THOMAS HAAS
Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/04982

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SUZUKI et al. Purine and purine alkaloid metabolism in Camella and Coffea plants. Phytochemistry Review Article Number 68. 1992, Vol. 31, No. 8, pages 2575-2584.	1-5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/04982

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C12N 9/00, 9/10, 15/00, 15/09, 15/29, 15/64, 15/82; A01H 1/00, 4/00, 5/00